Antagonistic effects of chemical mixtures on the oxidative stress response are silenced by heat stress and reversed under dietary restriction

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Abstract

Chemical agents released into the environment can induce oxidative stress in organisms, which is detrimental for health and has been linked to neurodegenerative diseases. *C. elegans* has been important as model organism to understand oxidative stress caused by chemical agents. In this work, we explore how chemical mixtures drive the oxidative stress response under various conditions. Our results indicate that mixtures drive responses differently than individual components, and that altering environmental conditions, such as increased heat and reduced food availability, result in dramatically different oxidative stress responses mounted by *C. elegans*. When exposed to heat, the oxidative stress response is diminished. Notably, when exposed to limited food, the oxidative stress response to juglone is significantly heightened, while interactions between some naphthoquinones components in mixtures cease to be antagonistic. This suggests that organismal responses depend on the environment and stressor interactions. Given the high number of variables under study, and their potential combinations, a simplex centroid design was used to capture such non-trivial response over the design space. This makes the case for the adoption of Design of Experiments approaches as they can greatly expand the experimental space probed in noisy biological readouts. Our results also reveal gaps in our current knowledge of the stress response, which can be addressed by employing sophisticated design of experiments approaches to identify significant interactions.

Introduction

Neurodegenerative diseases such as Huntington's , Alzheimer's, and Parkinson's disease are projected to increase in the global population (Caron et al., 2018; Rossi et al., 2018). Oxidative stress has been associated with these neurodegenerative diseases (Chege & McColl, 2014; X. Chen et al., 2016; Dias et al., 2013). Chemical agents that generate Reactive Oxygen Species (ROS) cause oxidative stress in organisms. Traditionally, oxidative stress by chemical agents have been studied by employing individual perturbations (Senchuk et al., 2017). However, the oxidative stress effects of chemical mixtures are poorly understood. Yet, realistic exposures include multiple components, while environmental factors have been directly implicated in altering the mechanisms by which chemicals induce toxicity and activate defense responses in living organisms (Vermeulen et al., 2020). It is thus pertinent to study how chemical mixtures drive oxidative stress, and how environmental conditions modify such responses.

Naphthoquinones are a class of compounds typically used as precursors of industrially relevant chemicals such as anthraquinone and dithianon, and are toxic in nature (Grolig & Wagner, 2000). The cytotoxicity and genotoxicity of individual naphthoquinones have been studied extensively (Fowler et al., 2018). These have been hypothesized to induce oxidative stress through two distinct mechanisms (Inbaraj & Chignell, 2004).They either cause depletion of glutathione, an important phase 2 detoxification enzyme, through Michael reaction; or they can also cause production of ROS through redox cycling (Inbaraj & Chignell,

2004). It is unclear if differences in the cytotoxic mechanisms amongst naphthoquinones could be reflected as differences in organismal responses to naphtaquinone mixtures.

C. elegans is a nematode that has proven to be a powerful model organism due to its small size, easy maintenance, and mapped genome and neuronal wiring. It enables *in vivo* studies using fluorescent markers due to their transparent bodies and ease of genetic manipulation (Corsi et al., 2015). They are also used as models to study neurodegenerative disease progression, among others, under the influence of genetic and environmental factors (Calahorro & Ruiz-Rubio, 2011). Previous studies on growth and fertility of *C. elegans* in the presence of fungicides and heavy metal mixtures have proven that they are compatible models to study chemical mixture toxicity (H. Chen et al., 2019; Lu et al., 2018; Vingskes & Spann, 2018; Wittkowski et al., 2019).

In *C. elegans*, the oxidative stress response pathway is activated to counteract the toxicity caused by oxidative stressors (Wu et al., 2017). SKN-1, a transcription factor ortholog to mammalian Nrf-2, is the major regulator of the oxidative stress response in *C. elegans* (Blackwell et al., 2015; Inoue et al., 2005; Przybysz et al., 2009). Activation of the SKN-1 pathway by chemicals and heavy metals, however, has been mostly studied by exposure to single chemicals (Benedetto et al., 2010; Hasegawa et al., 2008; Possik & Pause, 2015; Senchuk et al., 2017). Crombie et al. identified that heat stress inhibits the oxidative stress response mounted by *C. elegans* to juglone (Crombie et al., 2016). A two level full factorial design was used and the oxidative stress response was monitored through *gst-4* expression. *gst-4* encodes glutathione-S-transferase, a detoxification enzyme, which under the control of SKN-1 and is thus used as proxy for SKN-1 activity (Leiers et al., 2003; Link & Johnson, 2002; Tawe et al., 1998).

When assessing the effects of multiple variables, each factor is traditionally studied independent of the others through One-Factor-At-a-Time (OFAT) experiments (Goos & Donev, 2006). However, this method is not suitable for analysis of potential interactions between multiple conditions. Design of Experiments (DoE) is an approach where the input factors in experiments are systematically examined to determine the individual and combinatorial influence on the measured response (Goos & Donev, 2006; Goos & Jones, 2011). Full factorial design with multi-level factors for analyzing the effects of chemical mixtures results in unfeasible number of experimental runs to capture the response space. To compare the effects of different mixture proportions on the response, the total amount of oxidant mixture is constrained (Goos & Jones, 2011).

In this study, we analyzed the effects of naphthoquinone mixtures under various environmental conditions on the *C. elegans* oxidative stress response, as determined by a *gst-4* translational fluorescent reporter. We follow a DoE approach to minimize the number of experimental runs necessary to measure interactions between three naphthoquinones and two environmental conditions, while enabling comparisons from independent biological populations. We quantify response surfaces for these ternary mixtures under different conditions. We find that naphthoquinone mixtures drive antagonistic interactions, but these interactions are drastically modified by dietary restriction. On the other hand, heat stress abolishes oxidative stress response to both individual components and mixtures of naphthoquinones.

Materials and methods

Strain maintenance

C. elegans was maintained on standard Nematode Growth Medium (NGM) plates seeded with OP50 *E. coli* bacteria and maintained at 25ºC. The strain used in the experiments is CL2166: *dvLs19* [pAF15 (gst-4P:GFP::NLS)], which was obtained from the Caenorhabditis Genetics Center. Animals were exposed to either control (S-Medium) or naphthoquinone mixtures in liquid culture in the presence of HB101 *E. coli* as a food source, based on established procedures (Stiernagle, 2006). *E. coli* was grown in LB media in the presence of 4 mM streptomycin. Bacteria were washed thrice with SB media, pelletized, and resuspended

in S-Medium at a concentration of 100 mg/mL. Bacteria was then killed by heat treatment at 65ºC for a period of 45 mins (Crombie et al., 2016), to avoid bacterial metabolism to confound results.

Chemical preparation

Juglone, 1, 4-naphthoquinone, and plumbagin (**Fig. 1A**) were sourced from Fisher Scientific and stored according to supplier guidelines. 100 mM stock solutions were prepared for 1, 4-naphthoquinone and plumbagin by dissolving the powdered compounds in DMSO and stored at -20ºC. Juglone stock was prepared fresh before the start of each experiment, given its low stability (Senchuk et al., 2017).

Experimental design

An experimental design was generated to study the effect of oxidant mixtures at *ad libitum* feeding (2E10 cells/mL) and no heat stress conditions, using a simplex centroid design (7 points in the experimental space, **Fig. 1B**). The simplex centroid design was chosen as it entails a low number of experimental points, thus facilitating performing more replicates. This design entailed 40 runs split into 10 groups of 4 runs (where each design point had 5 – 7 replicates). To assign simplex centroid points to groups, we started from an I-optimal design generated in JMP Pro v 14.2 with 21 points in the experimental space, which already assigns each point to a group. These I-block points were then approximated to the closest point in the simplex centroid design, and their assignment to a group from the I-block design was maintained. An additional control run was tested along with each group to account for differences in populations due to any experimental variability (ambient temperature, humidity, etc). A population of animals from a single NGM plate was thus divided into 5 runs (4 for design points, and one as a control).

To study the effects of process conditions on responses induced by chemical mixtures, a split plot mixture experiment was performed. The whole plot factors were the two environmental stressors and the split plot factors were the naphthoquinone mixtures. Two levels of heat exposures: 20ºC and sublethal 33ºC were considered as the first environmental stressor. Two levels of HB101 bacterial diet regime - *ad libitum* (2E10 cells/mL) (AL) and dietary restriction (2E9 cells/mL) (DR) was considered as the second environmental stressor (Crombie et al., 2016; Keith et al., 2014). A total of 28 populations of animals were used for the split plot mixture experiments.

Each population was split into five runs. One run, at process conditions 20ºC and *ad libitum* diet named "overall control", was used to compare the 28 populations (7 points of simplex centroid design * 4 process conditions). Another run named "experimental control", was reserved for testing the effect of individual environmental stressor, i.e., a specific heat level and diet combination with no oxidant. Thus, each of the 4 process conditions were replicated 7 times across the 28 populations. The remaining three runs were exposed to the same combination of diet and heat exposure as the experimental control, but each was exposed to a different chemical mixture. These were assigned to groups similar to the previous experiment, using design experimental points from a simplex centroid. Hence, a total of 84 runs (7 points of the simplex centroid design * 3 replicates * 4 process conditions) and 56 control runs were used to study the effects of heat stress and dietary restriction on the naphthoquinone mixtures. The overall controls were plotted as Xbar and S control chart to ensure that populations were statistically comparable with each other.

Application of oxidative stress and fluorescence imaging

Approximately 30-40 worms were loaded into a well of a 24-well plate containing the oxidant and HB101 bacteria in S-Medium. Control experiments were conducted with an empty vector of 1% v/v DMSO. Animals were exposed to the oxidants for a period of 8 hours at a temperature of 20±1ºC. For Pgst-4::GFP imaging, worms were immobilized using a drop of 4 mM tetramisole on dried 2% agarose pads. Worms

were imaged using a wide field inverted fluorescence microscope Leica DMi8 at 5x magnification after the exposure period. A Spectra X laser illumination system centered at 470 nm was used for excitation, and a Hamamatsu Orca Flash 3.0 16-bit digital CMOS camera was used for image acquisition. A dose response to individual compounds was first quantified by exposing animals in increasing concentrations from 0 uM to 100 uM for a period of 8 hours. The naphthoquinone mixtures were applied based on the designs described the previous section.

Quantitative image processing

GFP driven by the *gst-4* promoter is observed throughout the animal, and its intensity was quantified to estimate oxidative stress response *in vivo*. Images were analyzed using a MATLAB script that generates a binary mask to identify a single worm per image. The MATLAB regionprops function was then used to quantify the mean intensity of the animal by overlapping the binary mask over the original image (**Fig. 1C**).

Statistical analysis

Data was compiled and analyzed using JMP Pro v.14.2. The standard least squares second order Scheffe model accounting for the individual components, mixture interactions, and effect of the process variables was used (Preece & Cornell, 1982). The block effect in the first design and the split plot in the second design were taken to be random effects. All effects, including interactions between the mixtures and process variables, with p value smaller than 0.05 during statistical testing ($\alpha < 0.05$) were considered as significant. Since this approach means dealing with multiple *C. elegans* populations (a population is considered animals cultured on the same NGM plate), we monitored both the mean and variability of gene expression to ensure that population responses are within statistical limits of each other. To assess whether different populations were comparable, we used control limits. A population plotted within the control limits is equivalent to failing to reject the null hypothesis of statistical control (i.e., all populations have equal means), and a population plotted outside the control limits is equivalent to rejecting the null hypothesis. The process mean was monitored using the X bar chart, while process variability was monitored using the S chart (Scouse, 1985). The responses of the controls were plotted using an Xbar (average) and S (standard deviation) control chart.

Results

Dose dependency of *gst-4* response

To identify relevant concentrations of chemical mixtures of plumbagin, 1-4-naphtaquinone, and juglone, (**Fig. 1A,B,C**) we first determined dose-dependent *gst-4* activity to individual components (**Fig. 1D**). Animals were exposed to individual compounds for a period of 8 hours, based on prior exposure studies with other compounds that suggest this time is sufficient to observe a *gst-4* response (Hasegawa et al., 2007; Van Raamsdonk & Hekimi, 2012). Worms exhibited a dose dependent *gst-4* activity to all 3 compounds. Concentrations around 200 μM are considered lethal (Hunt et al., 2011; Senchuk et al., 2017), and experiments were performed using a sublethal dose of 30 μM. Sublethal doses in the range of 20-30 μM for the 3 compounds under study are known to drive SKN-1-dependent expression of *gst-4* and also induce a hormetic effect driven by SKN-1 (Crombie et al., 2016; Hunt et al., 2011). As expected, we identified that as the naphthoquinones concentration increases, *gst-4* activity also increases and eventually saturates (**Fig. 1D**), as described in previous studies (Hasegawa et al., 2007; Van Raamsdonk & Hekimi, 2012). To assess the effect of mixture proportions on oxidative stress response it is necessary to fix the total naphthoquinone dosage. We ran the experiments at a combined total dosage of 30 μM, which allows studying the interactions of mixture components at different proportions without potential saturation of *gst-4* response.

Naphthoquinone mixtures show antagonistic effects under *ad libitum* feeding and physiological temperature conditions.

The first mixture experiments were performed under *ad libitum* feeding and no heat stress conditions based on the simplex centroid design. The average and standard deviations of the baseline *gst-4* responses of controls run for each of the ten sets of experiments were compared to determine if runs were comparable to each other. As shown in **Fig. 2A**, all measurements are within statistical control. We used a Scheffe model fit to develop a response surface (**Fig. 2B, Table S1**). The block effect added in the model indicates that populations 4, 6, and 10 exhibited a significant effect (**Table S2**), suggesting some experimental variation for these populations. However, these deviations caused by the block effects are accounted for in the model. The response surface indicates that the individual naphthoquinones induce a higher *gst-4* response than binary and ternary mixtures, suggesting an antagonistic interaction (**Fig 2B, Table S3**).

These unexpected antagonistic interactions could stem from different mechanisms of toxicity elicited by the mixture components. For instance, there are differences in the chemical reactivity of juglone and plumbagin based on electrochemical studies (Hernández-Muñoz et al., 2009). Naphthoquinones have been shown to result in toxicity through different mechanisms: redox cycling (which generates ROS) and direct Michael's addition (which depletes glutathione) (Hunt et al., 2011; Inbaraj & Chignell, 2004). Juglone has relatively higher chemical activity and could undergo Michael's addition to glutathione even at lower doses (Hernández-Muñoz et al., 2009; O'Brien, 1991). 30 µM of 1, 4-naphthoquinone and plumbagin could cause oxidative stress through redox cycling at lower doses, and only at higher doses cause Michael's addition to glutathione (Hernández-Muñoz et al., 2009; Seshadri et al., 2011). Mixtures of these chemicals could thus result in lower *gst-4* activation than individual mixtures, if *gst-4* induction is more sensitive towards one of these toxicity mechanisms.

It has also been shown that there are differences in oxidant detection in *C. elegans.* For instance, sublethal levels of H_2O_2 activate the I2 neuron, while paraquat only elicits a response at a very high concentration (Bhatla & Horvitz, 2015). The difference in the *gst-4* response to individual compounds and mixtures could stem from the combined effects of differences in xenobiotic detection and the mechanisms of toxicity by the different naphthoquinones. This result indicates that individual components do not act through a singular mechanism, and that mixtures can drive significantly different organismal responses than individual components, even for highly similar chemical species.

Naphthoquinone mixtures induce different responses under different environmental conditions

Temperature and dietary intake have been shown to affect the oxidative stress response in *C. elegans* (Crombie et al., 2016; Paek et al., 2012; Przybysz et al., 2009), and altering environmental conditions can change the response mounted by the organism (Paek et al., 2012). To study their effect on the oxidative stress response, we tested how exposure to heat stress and dietary restriction modified the *gst-4* response to naphthoquinone mixtures. We used the extracted quantitative *gst-4* expression data to fit a mixed effects model (**Table S4**). The overall control was plotted as Xbar and S chart and it indicates that the populations are within statistical limits of each other (**Fig. 3B**). Block effects analysis revealed population 5 exhibited a statistically significant effect compared to the other 27 populations (**Table S5**). However, the model corrects for the effect caused by the treatment application process on population 5.

Heat stress and dietary restriction significantly affect the oxidative stress response, inducing a lower baseline *gst-4* response than overall controls of 20ºC and *ad libitum* conditions (**Fig. 3A**). The reduction in *gst-4* expression levels by heat stress could be explained by organismal prioritization of the heat shock response over the oxidative stress response, as previously suggested by Crombie et al. (Crombie et al., 2016). The reduction in *gst-4* levels by dietary restriction could be the result of the reduced activity of *cct-*

4 under dietary restriction, which encodes a chaperonin directly involved in SKN-1–dependent transcription of *gst-4* (Rollins et al., 2019).

Naphthoquinone mixtures induce a differential response in *gst-4* under different process conditions (**Fig. 3C-E, Fig. S1, Table S6**). The *ad libitum* and 20ºC response surface is the repetition of the first mixture experiment (**Fig. 2B, Table S7**). As we increased the temperature to 33ºC, heat stress reduces the *gst-4* response to pure components and mixtures. This is consistent with previously observed results (Crombie et al., 2016), and has been attributed to prioritization of proteostasis over detoxification., where the HSF-1 driven heat stress response genes are upregulated to prevent protein misfolding (Crombie et al., 2016). Neither mixtures of oxidants nor individual components elicit a *gst-4* response under this heat stress condition (**Fig. 3E**).

As mentioned above, dietary restriction induces a reduction in baseline *gst-4* (**Fig.** 3A), potentially due to down regulation of *cct-4* chaperonin (**Fig. 3A**) (Rollins et al., 2019). In contrast, reduced food concentration did not affect the *gst-4* response to 1,4-naphthoquinone and plumbagin (**Fig. 3E**) and it drastically increased the activity to juglone, as compared to *ad libitum*. Furthermore, exposure to binary or ternary mixtures under dietary restriction did not result in antagonistic interactions observed in mixtures at *ad libitum* dietary regime. Interestingly, although under *ad libitum* condition, individual components drive *gst-4* expression at equivalent levels, dietary restriction-induced responses show high specificity for juglone, suggesting differences in organismal processing of similar oxidants. A possible explanation could be the cross-regulation between the diet regulated DAF-16 and oxidative stress regulated SKN-1 pathways (Benedetto et al., 2019; Tullet, 2015). This crosstalk could lead to the upregulation of *gst-4* under simultaneous dietary restriction and oxidative stress where differences in activity depends on the specific oxidative stressors (**Fig 3E**). It is unclear, however, why these DR-effects would be specific to the oxidant type. Potentially, these differences could stem from differences in toxicity and detection mechanisms, as explained before, coupled with multiple transcriptional pathways interacting at the organismal level. When both DR and heat stress were applied, the heat stress response still dominated, and interactions with dietary restriction seen at 20ºC were lost. Further, experimentation tracking other detoxification genes, and activity of key transcription factors, is necessary to understand the differences in toxicity mechanisms of naphthoquinone mixtures under the different process conditions.

Discussion

In this work, we took advantage of a design of experiments approach to study the oxidative stress response driven by combinatorial exposures to naphthoquinones in a variety of environmental conditions. Unlike traditional full-factorial designs, which would entail an unfeasible number of experiments, using block effects and a simplex centroid design enabled performing experiments in a non-simultaneous and feasible manner. Using mixed second order Scheffe model statistical analysis, we built response curves to ternary naphthoquinone mixtures in a variety of environmental conditions, which revealed significant interactions between chemicals. Surprisingly, these interactions are drastically modulated by environmental conditions such as temperature and food availability. Antagonistic interactions were abolished by heat stress, while dietary restriction heightened the response to specific oxidative stressors. These findings highlight the importance of experimental analysis in realistic settings, where a variety of chemical components are present, and where environmental conditions vary significantly. In addition, the identified interactions between naphthoquinone mixtures, heat stress, and dietary restriction, shed light on organismal integration and processing of stressors and environmental factors.

C. elegans are complex in nature and many factors could affect the response to oxidative stress such as variation in developmental period, food availability on plate, temperature, as well as biological stochasticity. It is necessary to control and account for such effects as combinatorial mixture experiments cannot be performed using a single population and must be distributed across different populations. Control

charts have proven to be useful in plotting the response of controls and identifying potential problematic populations, which might show a different response. Such populations can have different biological activity and can affect the results of the experiments being performed. The mixture experiments performed with such populations can be repeated to ensure that the response recorded is only due to the mixture effects. Adding block factors and split plots as random effects to the model also help us compare between populations, even if these exhibit differences that could come from experimental or biological variation.

Thus, using a combination of mixture experiments and control charts we have highlighted the differential *gst-4* response by *C. elegans* to oxidative stressor mixtures under different environmental conditions. These results warrant further investigation of the different transcriptional pathways involved, and shed light on how organisms respond to variable environments and realistic chemical exposures.

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Competing interests:
The authors declare no competing interests.

Author contributions:
All authors designed the experiments and conceived the work. KSA and JH carried out experiments and analyzed data. JWS and KSA performed design of experiments and statistical analysis. KSA, JH, and ASM wrote the manuscript.

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Data availability:
All data underlying this work can be requested from the authors.

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Figures

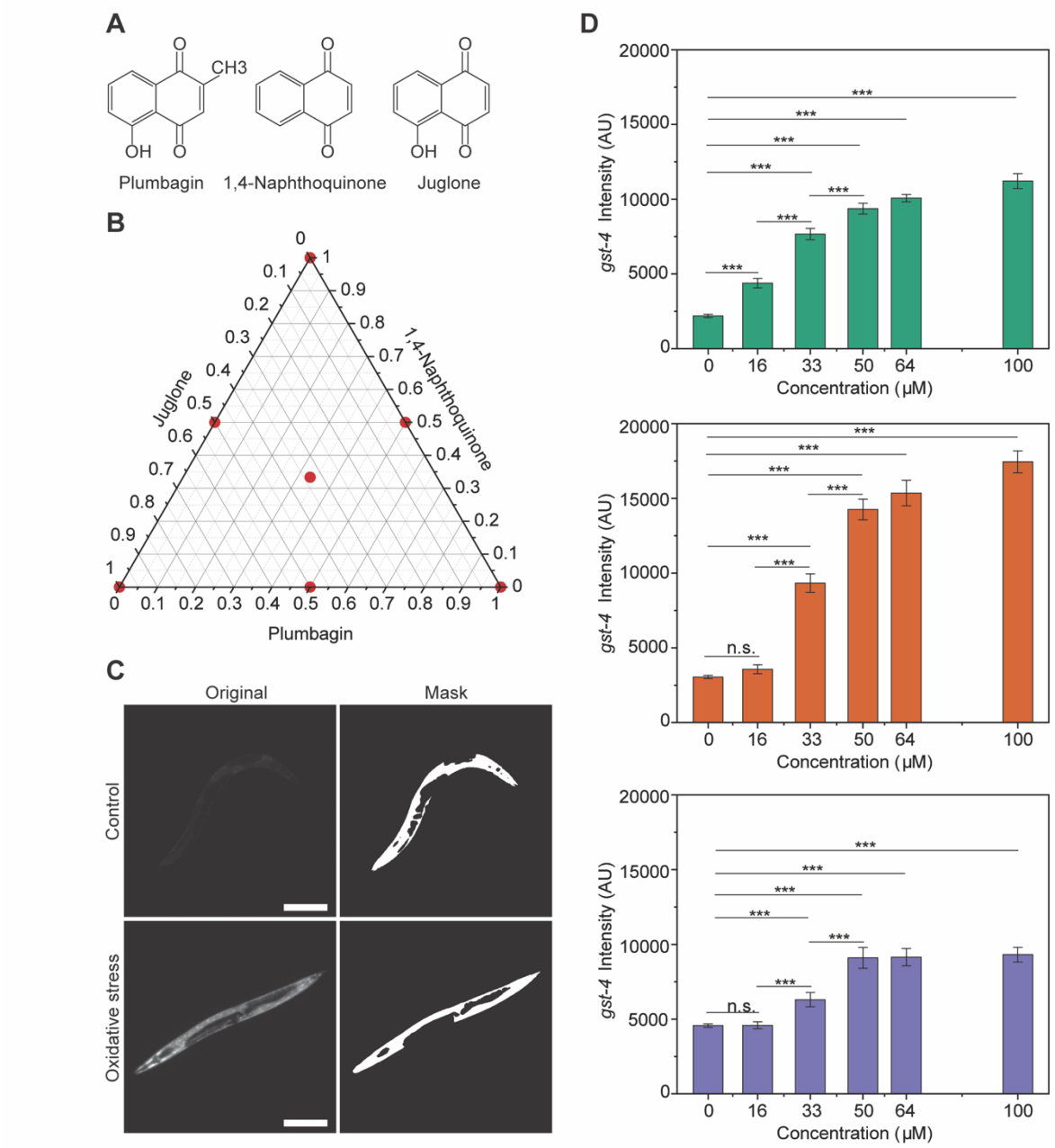

Figure 1: *gst-4* response is dose dependent. A) Compounds used for oxidative assays. B) Simplex centroid design. C) CL2166 worms under control and oxidative stress with corresponding masks for extracting *gst-4* expression levels. D) gst-4 dose dependent response to Plumbagin, 1, 4-Naphthoquinone, and Juglone (top to bottom). $p > 0.05$ (n.s.), $p < 0.001$ (***). Error bars are SEM. All p-values were calculated using Tukey HSD for all pairwise comparisons after one-way ANOVA (Unequal Variances) comparison in JMP 14.2. Scale bars are 200 μm.

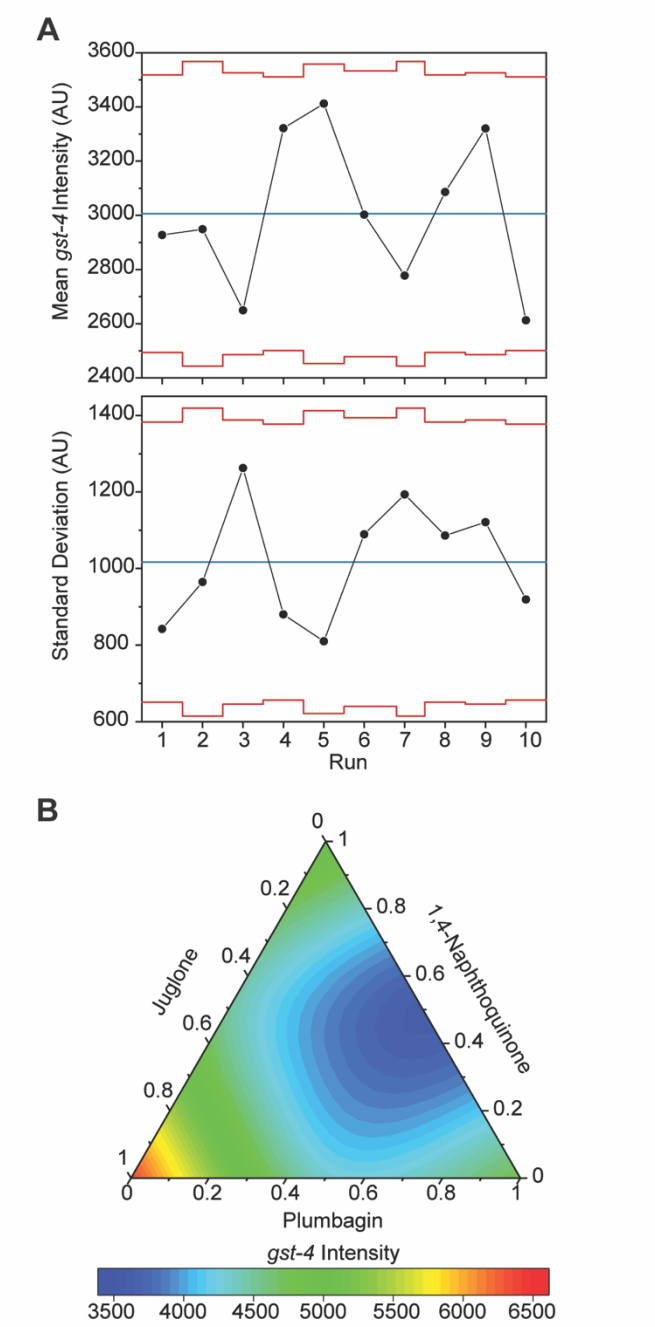

Figure 2: Naphthoquinone mixtures show no synergistic effects under *ad libitum* feeding and no heat stress conditions. A) Xbar and S-chart for 10 populations used for oxidative stress assays. B) Response surface of *gst-4* expression levels for CL2166 animals under oxidative stress, 20°C, and *ad libitum* feeding. Response surface modeled using standard least squares second order Scheffe model where main effects and interactions were tested for significance (Table S3).

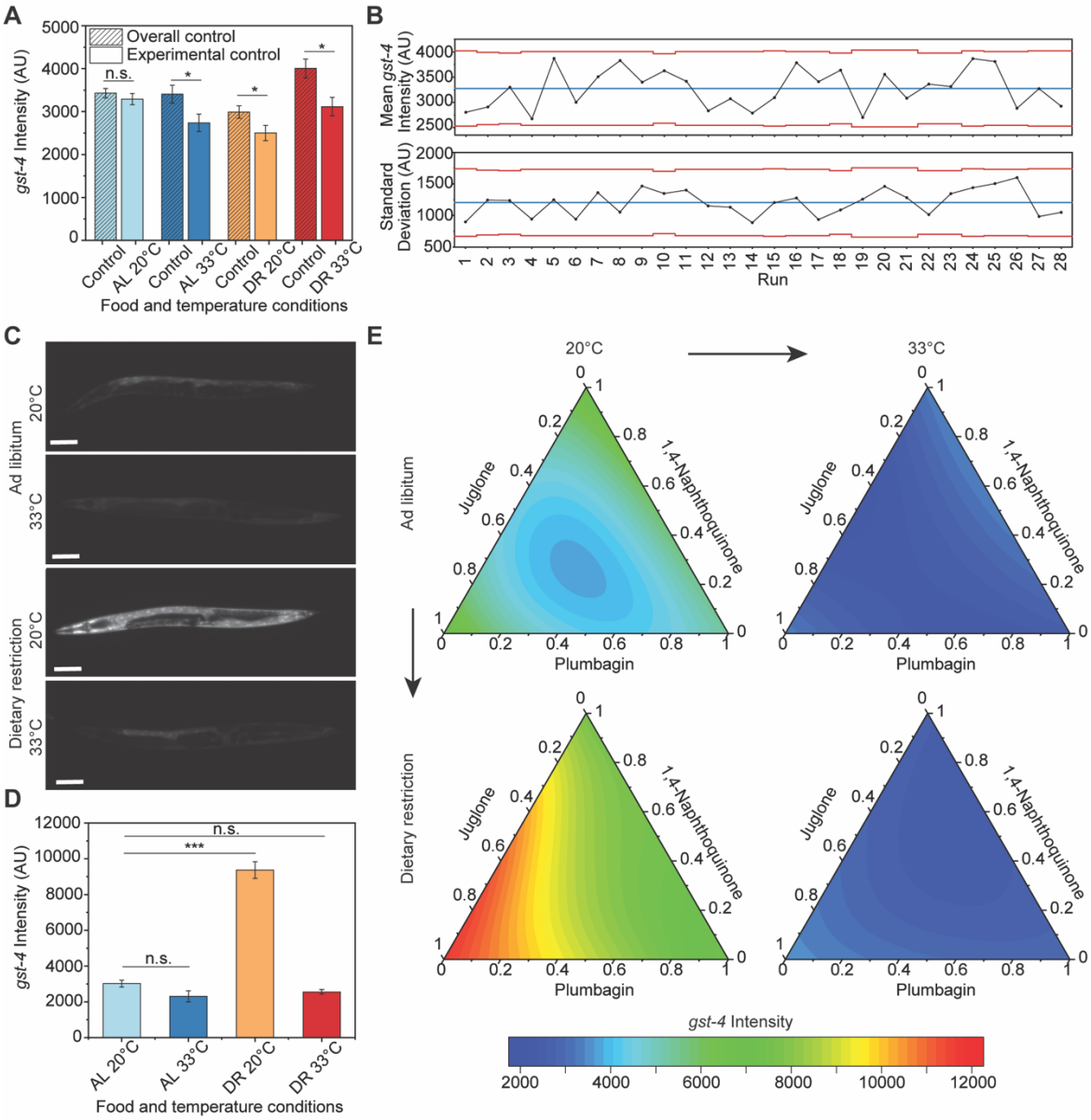

Figure 3: Naphthoquinone mixtures show differential response under simultaneous stress exposure. A) Testing for main effect of temperature and food concentration. B) Xbar and S-chart for 28 populations used for oxidative stress assays. C) Representative CL2166 animals under oxidative stress (1,4-Naphthoquinone*Juglone*Plumbagin mixture) at different environmental conditions. D) *gst-4* expression levels for the representative CL2166 animals under oxidative stress mentioned in 3C. E) Response surface of *gst-4* expression levels for CL2166 animals under oxidative stress at different environmental conditions. $p > 0.05$ (n.s.), $p < 0.05$ (*). All p-values were calculated using t-test in JMP 14.2. Response surfaces modeled using standard least squares second order Scheffe model where main effects and interactions were tested for significance (Table S6). Scale bars are 100 µm. The experimentally acquired data is represented as conventional bar plots in Fig S1.